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Genetic diversity analysis of chewing sugarcane (*Saccharum officinarum* L.) varieties by using RAPD markers

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ABSTRACT

In the present study an efficient and easy method was followed for the isolation of DNA from meristem cylinder in five chewing sugarcane varieties, namely Amrita, Bomaby, Babulal (Co.527), Q83 and Misrimala. The quality and quantity of DNA were assured by visual estimation using agarose gel electrophoresis and UV spectrophotometry. The highest amount of DNA was retrieved from the Amrita (3250 ng/ml) and the lowest amount was attained from the variety Q83 (1450 ng/ml). The amount of recovered DNA was enough for PCR amplification and marker studies such as random amplified polymorphic DNA (RAPD). Using RAPD markers, bands obtained from fingerprinting (190 bp to 1200 bp) showed 73.5% polymorphism. The dendrogram, based on linkage distance using unweighted pair group method of arithmetic means (UPGMA), indicated segregation of the five chewing varieties of sugarcane into two main clusters. Amrita, Bombay and Misrimala were grouped in cluster 1 (C1) followed by sub-clusters. Babulal and Q83 were grouped in cluster 2 (C2). The results of the present investigation also revealed that the twenty RAPD primers were able to identify and classify the chewing sugarcane varieties based on their genetic relationship.

Key words: genetic diversity, RAPD, PCR, UPGMA, *Saccharum officinarum*

Introduction

Sugarcane (*Saccharum officinarum* L.) is one of the leading crops in the world and it is the second most important cash crop, especially for the North and Southern part of Bangladesh. It provides cheap food in the form of "sugar" and "gur (zaggary)". It is also a food crop as people use to chew and drink sugarcane juice. Northern, Southern and Central region of Bangladesh practice sugarcane cultivation and commands 2% area of total cultivated land. Twenty-five lakh metric tons (MT) from the total production of sugarcane are used for sugar production, 30 lakh MT for gur, 10 lakh MT for chewing purposes. Globally, it occupies less than 2% of the total cropped area, producing 1350 million MT of cane (FAO, 2004). DNA isolation, qualification and quantification are the prerequisite for DNA fingerprinting and genetic diversity study of varieties based on molecular markers. The genomic DNA is the base material for molecular studies. DNA genetic markers form the basis of current strategies for genome analysis, gene mapping and germplasm identification. However, DNA markers seem to be best candidates for efficient evaluation and selection of plant material. Estimation of genetic variation increasingly are being based upon information at the DNA level by various molecular markers such as, Randomly amplified polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length polymorphism (RFLP), Simple Sequence Repeat (SSR) (Hossain et al., 2012; Sajib et al., 2012). Among them, RAPD is a PCR based technique for identifying genetic variation.

RESEARCH ARTICLE

RAPD analysis has been used extensively for various purposes, which include genetic diversity analysis (Yu & Nguyen, 1994). RAPD analysis (Williams et al., 1990) is simple, quick, easy to perform, requires small amount of DNA for analysis and major advantage is that no prior sequence information is required. These benefits justify the frequent application of the technique in genetic variability studies (Mondal & Chand, 2002; Bennici et al., 2004; Feuser et al., 2003). Therefore, the present investigation was undertaken for fingerprinting of five chewing sugarcane varieties using RAPD markers.

Materials and Methods

Plant materials

Five chewing sugarcane varieties (Amrita, Bombay, Babulal (Co. 527), Q87 and Misrimala) from Bangladesh Sugarcane Research Institute (BSRI), Ishurdi, Pabna, Bangladesh, were used as experimental plant materials.

Isolation of genomic DNA

A modified method of Aljanbi et al. (1999) has been used to isolate the total genomic DNA from meristem cylinder of five selected chewing sugarcane varieties. The isolated DNA (2 µl) was loaded on 1% agarose gel to detect the quality of the DNA. The quantity and quality of DNA was also determined by absorbance reading at 260 nm and 260/280 nm absorption ratio respectively. Finally the isolated DNA was diluted with TE buffer to get the final concentration at 50 ng/µl.

PCR amplification and electrophoresis

Twenty primers (markers) of random sequence (Operon Technologies, Inc., Alameda, California, USA) (Table 1) were used for the whole sample set of five chewing varieties of sugarcane. PCR amplification was done in an oil-free thermal cycler (Genius, Techne, Cambridge Limited) following the PCR profile of 94ºC for 3 minutes (initial denaturation) followed by 40 cycles of 1 minute denaturation at 94ºC, 1 minute annealing at 35ºC and elongation or extension at 72ºC for 2 minutes. After the last cycle, a final step of 7 minutes at 72ºC was added to allow the complete extension of all amplified fragments. After completion of cycling programme, the reactions were held at 4ºC. PCR amplification was carried out with 1 µl Buffer A (10X) with 15 mM $MgCl_2$, 1 µl of 2.5 mM dNTPs, 100ng template DNA, 0.2 µl of *Taq* DNA polymerase (5 U/µl) (Bangalore Genei Pvt. Ltd., India), suitable amount (3.3 µl) of sterile de-ionized distilled water and 2.5 µl of RAPD primer from 10 µM working solution. PCR products from each sample were confirmed by running 1.5% agarose gel containing 13 µl ethidium bromide (10 mg/ml) in 130 ml 1X TBE buffer at 120V for 1.5 hours. Loading dye (2 µl) was added to the PCR products and loaded in to the wells. A molecular weight marker DNA (100 bp DNA ladder) was also loaded on either side of the gel.

RAPD data analysis

Following electrophoresis, all distinct bands or fragments were thereby given identification numbers according to their position on the gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer. The scores obtained using all primers in the RAPD analysis were then combined to create a single data matrix. This data were used for estimating linkage distance (D) and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among the populations using computer program "Statistica". Genetic similarity values were determined according to the following formula:

$$\text{Similarity index (SI)} = 2N_{xy}/ N_x + N_{y.}$$

where, N_{xy} is the number of RAPD bands shared by individuals x and y respectively, and N_x and N_y are the number of bands in individuals x and y, respectively (Lynch 1990, Wilde et al., 1992).

Results

Qualification and quantification of genomic DNA

The purity and quality of the isolated DNA was also determined by using 260/280 nm UV absorption ratios. In this investigation, the range of A260/A280 ratio among five chewing sugarcane varieties are from 1.84 – 1.96, which indicate the good quality of DNA (Table 2). The visual estimation of DNA was done by agarose gel electrophoresis (1%) comparing with the known molecular weight marker Lambda DNA *Hind*III digest (Figure 1). In the spectrophotometic method, the amount of DNA was determined by taking absorbance reading at 260 nm. In this study, the recovered amount of DNA was ranged from 1450-3250 ng/µl. The highest amount of DNA was recovered from the variety Amrita (3250 ng/µl) and the lowest amount was obtained from the variety Q83 (1450 ng/µl). The concentration of DNA is presented in the Table 2.

RESEARCH ARTICLE

Table 1: *List of 20 primers used for RAPD analysis.*

Primer code	Sequence (5′-3′)	G+C content (%)	Primer code	Sequence (5′-3′)	G+C content (%)
OPA-01	CAGGCCCTTC	70%	OPB-18	CCACAGCAGT	60%
OPA-02	TGCCGAGCTG	70%	OPB-20	GGACCCTTAC	60%
OPA-03	AGTCAGCCAC	60%	OPC-01	TTCGAGCCAG	60%
OPA-06	GGTCCCTGAC	70%	OPC-02	GTGAGGCGTC	70%
OPA-08	GTGACGTAGG	60%	OPC-03	GGGGGTCTTT	60%
OPA-12	TCGGCGATAG	60%	OPC-04	CCGCATCTAC	60%
OPB-05	TGCGCCCTTC	70%	OPD-01	ACCGCGAAGG	70%
OPB-09	TGGGGGACTC	70%	OPD-03	GTCGCCGTCA	70%
OPB-11	GTAGACCCGT	60%	OPE-02	GGTGCGGGAA	70%
OPB-15	GGAGGGTGTT	60%	OPE-04	GTGACATGCC	60%

Table 2. *The ratio of spectrophotometric absorption readings of different samples at 260 nm and 280 nm for determination of the DNA quality.*

Serial No	Chewing sugarcane varieties	Absorbance ratio (A260/A280)	Concentration of DNA (ng/µl)
1	Amrita	1.86	3250
2	Bombay	1.87	2900
3	Babulal (Co. 527)	1.96	2450
4	Q 83	1.93	1450
5	Misrimala	1.84	1750

PCR amplification and electrophoresis

DNA fingerprinting of the five chewing sugarcane varieties was performed using twenty RAPD primers (Table 1). The size of amplification products were estimated by comparing the migration of each amplified fragment with that of a known size fragments of molecular weight marker (100 bp DNA ladder). Among the twenty primers eight primers (OPA-02, OPA-08, OPA-12, OPB-05, OPB-18, OPC-02, OPC-04, and OPE-04) produced comparatively maximum number of high intensity bands with minimal smearing and they exposed band sizes that ranged from 190 bp to 1200 bp (Table 3). Representative electrophoregrams according to primers OPB-05, OPB-18 and OPC-04 are shown in Figure 2. The highest number of bands (2.00) per variety was amplified from the primer OPC-04.

Polymorphism and intervariety similarity revealed by RAPD markers

A total number of 53 bands were obtained from the amplicons using eight primers varied from 3 (OPA-02) to 10 (OPC-04) (Table 3). Out of the 53 bands, 16 were monomorphic and 37 bands were polymorphic. The primer OPA-08 produced 8 polymorphic bands and it showed the highest level of polymorphism (100%). Approximately 73.50% were polymorphic with 8 RAPD markers used among the 5 chewing sugarcane varieties. The intervariety similarity indices between Babulal and Q83 was found to be the highest (66.67%). The lowest (0%) intervariety similarity indices were observed between Amrita and Bombay, Amrita and Babulal, Amrita and Q83, Bombay and Babulal, Bombay and Q83, Babulal and Misrimala, Q83 and Misrimala. Among five chewing sugarcane variety the similarity (S_i) was 8.80%

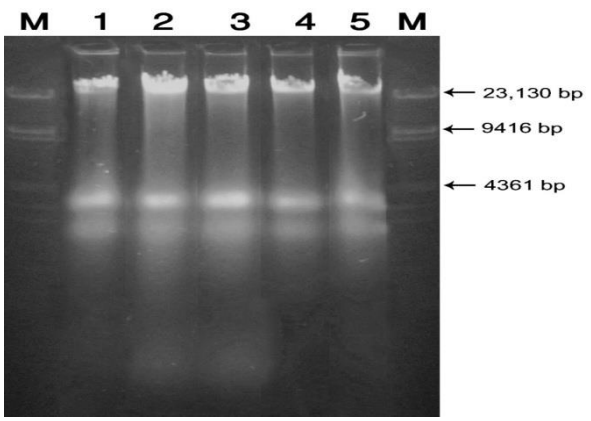

Figure 1: *Electrophoregram of ethidium bromide stained genomic DNA samples of five chewing sugarcane varieties (M = Lambda DNA with Hind III double digest, Lane 1 = Amrita, Lane 2 = Bombay, Lane 3 = Babulal, Lane 4 = Q83, Lane 5 = Misrimala.*

RESEARCH ARTICLE

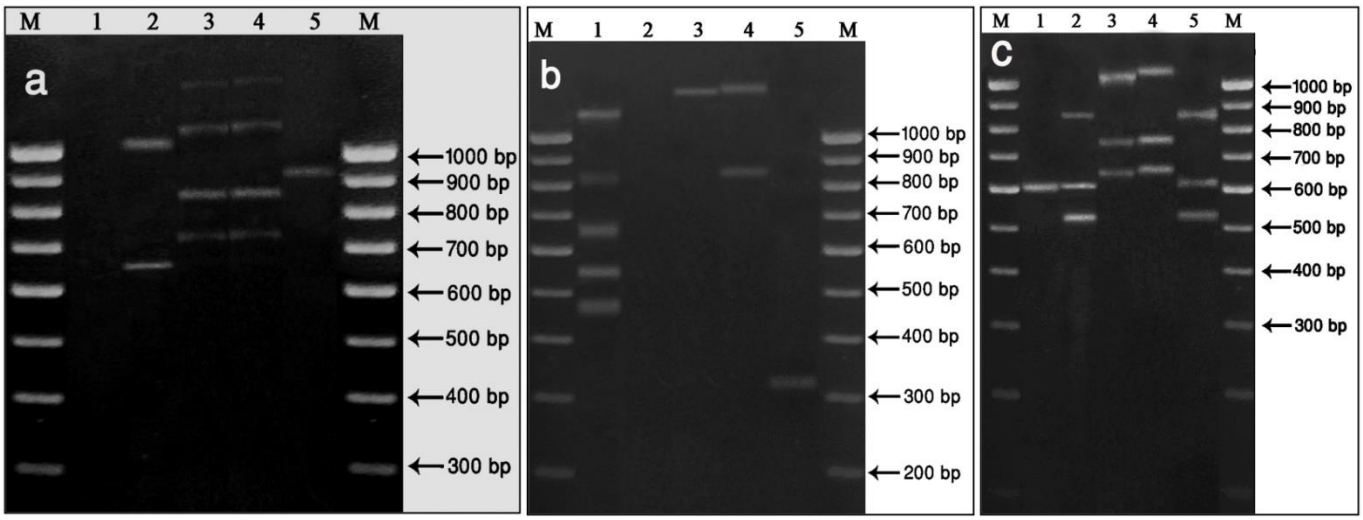

Figure 2. *DNA fingerprinting of five chewing sugarcane varieties based on RAPD primers through 1.5% agarose gel, (a) OPB-05 (b) OPB-18 (c) OPC-04. Lane M: Molecular weight marker (100bp DNA ladder); Lane 1: Amrita; Lane 2: Bombay; Lane 3: Babulal; Lane 4: Q 83; and Lane 5: Misrimala.*

Table 3. *List of scored RAPD primers with corresponding bands, their size ranges, number of monomorphic and polymorphic bands, polymorphism and number of band per variety in five chewing sugarcane varieties.*

Primer codes	Size ranges (bp)	Total number of bands scored	Number of monomorphic bands	Number of polymorphic bands	Polymorphism (%)	Number of band per variety
OPA-02	300-350	03	0	03	100.00	0.6
OPA-08	190-395	08	0	08	100.00	1.6
OPA-12	490-1200	07	02	05	71.43	1.4
OPB-05	650-1200	08	05	03	37.50	1.6
OPB-18	310-1200	07	01	06	85.71	1.4
OPC-02	400-1100	06	04	02	33.33	1.2
OPC-04	500-1050	10	04	06	60.00	2.0
OPE-04	480-700	04	0	04	100.00	0.8
Total		53	16	37		
Average		**6.63**	**2.00**	**4.63**	**73.50**	

Linkage distances between the varieties

The values of pair-wise comparisons of linkage distances (D) analyzed by using computer software "Statistica" between varieties were ranged from 14.0 to 33.0 (Table 4). The highest linkage distance (33.0) was found in Babulal *vs*. Misrimala variety pair and the lowest linkage distance (14.0) was found in Babulal *vs*. Q83 variety pair.

Pattern of clustering

The dendrogram based on linkage distance using UPGMA clustering indicated segregation of the five chewing verities of sugarcane into two main clusters at linkage distance 30 (Figure 3). Amrita, Bombay and Misrimala were grouped in cluster 1 (C_1). Babulal and Q83 were grouped in cluster 2 (C_2). In cluster 1, Bombay and Misrimala formed a subcluster 1 (SC_1) and Amrita alone formed another sub-cluster 2 (SC_2). Again, the verities of subcluster 1, Bombay and Misrimala were grouped together with lower linkage distance of 15.

RESEARCH ARTICLE

Table 4. *Summary of linkage distances values for different cultivar pairs of chewing sugarcane.*

Varieties	Amrita	Bombay	Babulal	Q83	Misrimala
Amrita	0.0	16.0	32.0	30.0	19.0
Bombay	16.0	0.0	28.0	26.0	15.0
Babulal	32.0	28.0	0.0	14.0	33.0
Q83	30.0	26.0	14.0	0.0	31.0
Misrimala	19.0	15.0	33.0	31.0	0.0

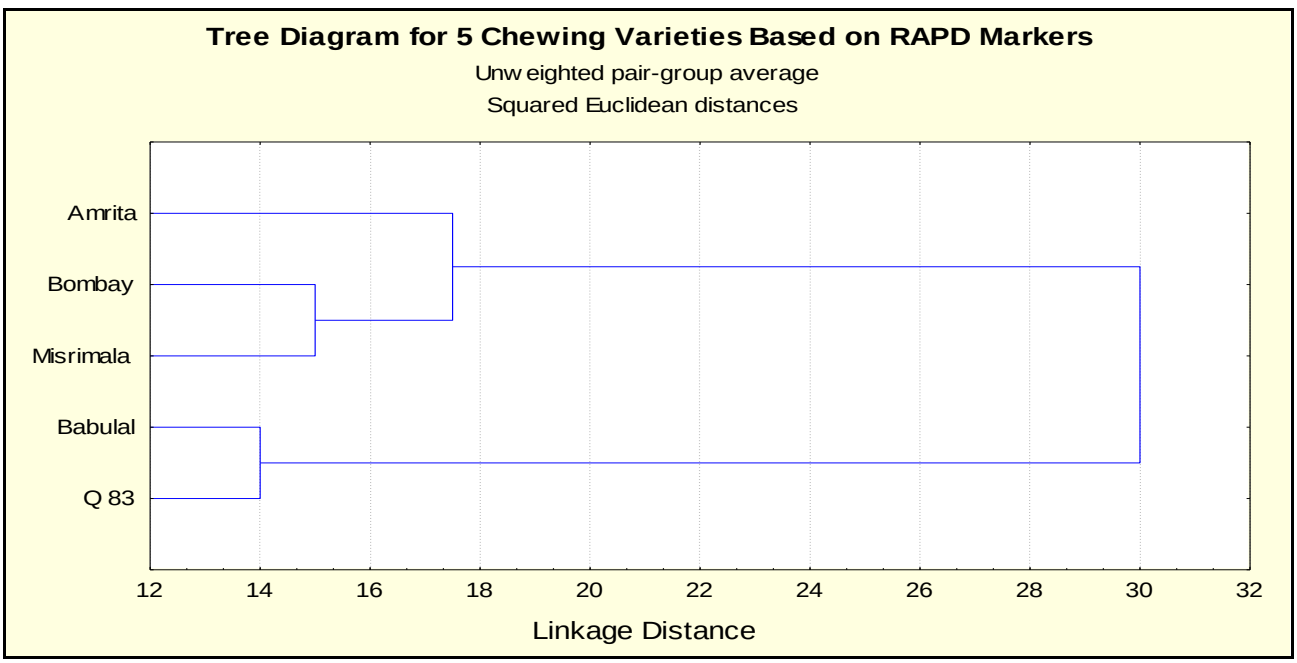

Figure 3. *UPGMA cluster analysis based on RAPD markers showing genetic relationship among chewing sugarcane varieties.*

Discussion

DNA isolation, qualification and quantification are the prerequisite for DNA fingerprinting and genetic diversity study of varieties based on molecular markers. In 1999, Aljanabi et. al. reported the range of A260/A280 ratio from 1.76 – 1.96 for good quality DNA of sugarcane. In this investigation, this range was 1.84 – 1.96, which indicate that the quality of isolated DNA was good. The recovered amount of DNA was higher (1450-3250 ng/µl). This was perhaps due to efficiency of the method. A basic understanding of the genetic diversity that exists in the germplasm available for breeding is essential to the success of a breeding program. This knowledge is useful in the utilization and management of genotypes and indeed genes in the breeding gene pool. Rapid advances in the field of molecular biology and its allied sciences made the use of molecular markers a routine practice providing plant breeders a precise tool in analyzing genetic diversity for plant improvement. For achieving improved productivity in sugarcane crops, it is essential to maintain a high degree of genetic diversity among the commercial varieties and breeding populations (Tabasum et al., 2010). Among the different molecular techniques, RAPD is widely used to study the variation at DNA level among the variants (Orapeza et al., 1995; Rout et al., 1998; Soniya et al., 2001; Geisteira et al., 2002; Bennici et al., 2004). In this study, genetic analysis was performed using RAPD markers because this method does not require knowledge of the sequence of the DNA under study (Wolfe & Litson, 1998). The most attractive feature of RAPD analysis is that it can be

RESEARCH ARTICLE

used on pooled DNA samples to rapidly screen for linked DNA markers (Michelmore et al., 1991). Therefore, the present investigation reported that out of 20 primers only 8 primers enabled the identification of polymorphism among variants of sugarcane. Using RAPD markers, as high as 73.50% polymorphic bands were detected in 5 chewing sugarcane varieties. Finally, this study will facilitate the use of RAPD-PCR based fingerprinting in marker assisted applications in sugarcane breeding. Results from cluster analysis and linkage distance (D), indicate that Amrita is different from other varieties used in this study. It is well experienced that field performances of variety Amrita is superiorly different from other cultivated varieties based on different criteria, such as sweetness of cane, rind softness, external appearances, fiber softness etc. Information on the genetic relationships among varieties based on the molecular markers can help to decide which variety is highly variable. Plant breeders could get the idea of breeding program and goal directed towards the selection of diverse parents to produce heterotic hybrid varieties.

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